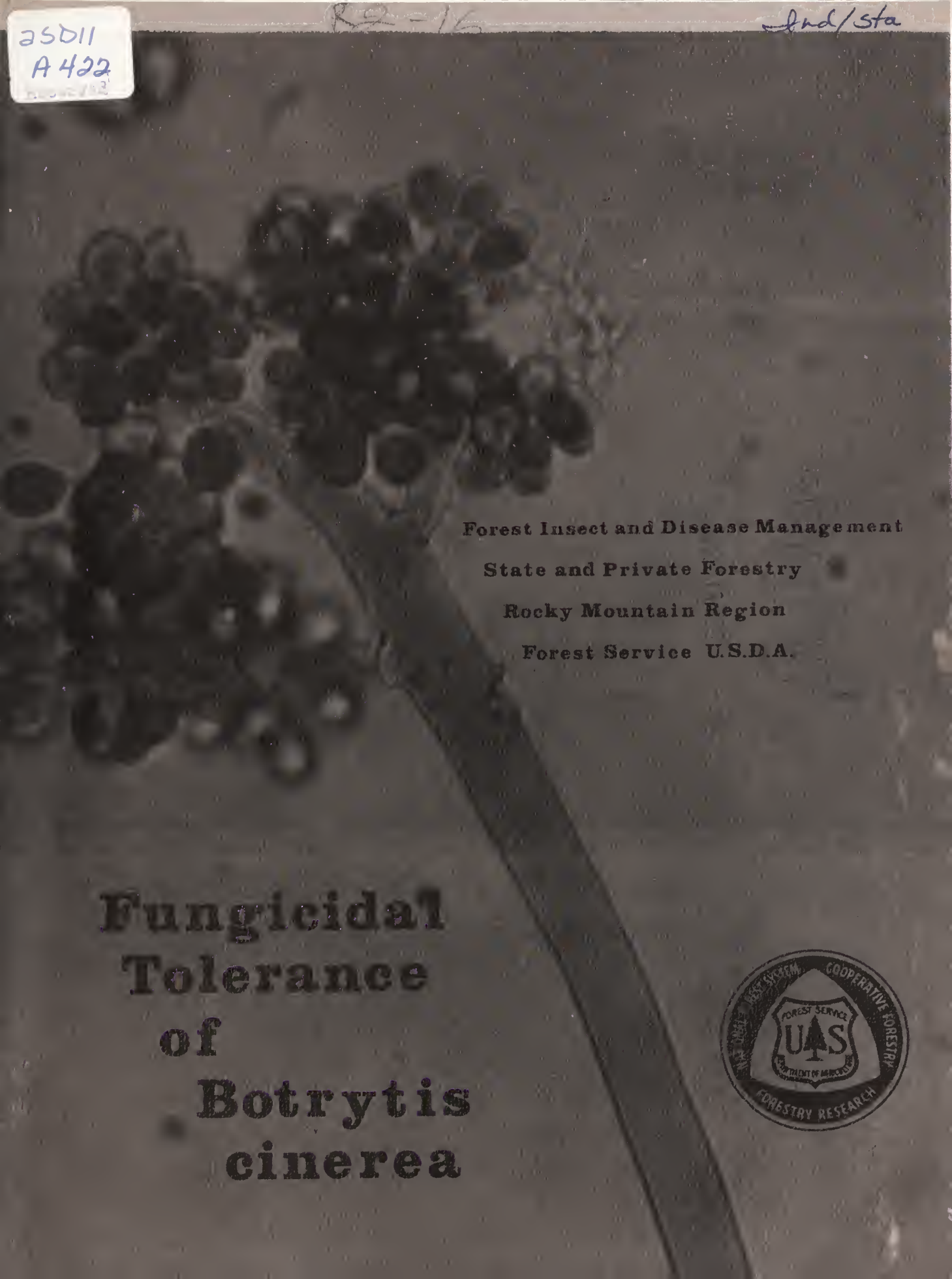

Forest Insect and Disease Management
State and Private Forestry
Rocky Mountain Region
Forest Service U.S.D.A.

Fungicidal Tolerance of Botrytis cinerea

FUNGICIDAL TOLERANCE OF *Botrytis cinerea*

by

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Technical Report R2-16

October 1978

Forest Insect and Disease Management
State and Private Forestry
Rocky Mountain Region
USDA Forest Service
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ACKNOWLEDGEMENTS

The authors acknowledge assistance of A. H. McCain for providing benomyl-tolerant and sensitive isolates and T. D. Landis, F. Rothe, and M. Strachan for providing conifer seedling material from which pathogen isolates were obtained. We are grateful to C. D. Minnemeyer for assistance with data analysis and D. W. Johnson, W. Nishijima, G. Peterson, and J. Hopkins for manuscript review.

ABSTRACT

Numerous *Botrytis cinerea* isolates from containerized conifers in three Colorado nurseries exhibited tolerance to commercially-used fungicides. Some isolates were tolerant to benomyl, captan, chlorothalonil, mancozeb, and zineb during growth tests on agar medium. Dichloran was the only fungicide that completely inhibited all isolates tested. Benomyl at 50 ppm did not inhibit spore germination on agar, although germ tube growth of sensitive isolates was restricted. These results indicate that the *B. cinerea* isolates tested have tolerance to commercial fungicides. Prescriptions for fungicide application should be based on tolerance characteristics of isolates present.

INTRODUCTION

Grey mold of conifers, caused by the fungus *Botrytis cinerea* Pers. ex Fr., may become a problem in containerized production in greenhouses (17, 21). The fungus is usually a saprophyte on necrotic tissues (7, 14, 15); however, under conditions promoted by overcrowding and overhead irrigation, it may attack healthy tissues and kill seedlings (17).

Despite regular application of fungicides to control *B. cinerea* in greenhouses (21), numerous reports (8, 16, 17, 23, 24) indicate tolerance of the fungus to these chemicals. Multiple-fungicide tolerance by this and other fungi has been reported (1, 24). However, many reports (2, 3, 9, 20, 22) emphasize that fungi tolerant to one fungicide are often sensitive to other chemicals.

Mortality of containerized lodgepole pine caused by *B. cinerea* (Figs. 1 & 2) was recently found at Colorado Hydroponics Nursery (Lyons, Colorado). Bi-weekly application of benomyl did not control the disease. Benomyl had not been used previously in the greenhouses.

Low levels of *B. cinerea*-induced mortality have occurred over a number of years at the Colorado State Forest Service Nursery (Ft. Collins, Colorado) despite regular benomyl applications. At Mt. Sopris Tree Nursery, USDA Forest Service (Carbondale, Colorado), losses due to grey mold occurred within one year on the second crop of conifers in a new greenhouse. Routine benomyl application was practiced during that year in the greenhouse.

Because of occurrence of *B. cinerea*-induced mortality despite regular application of benomyl, tests were conducted to evaluate fungicidal tolerance in strains of the fungus from each of these three Colorado nurseries.

MATERIALS AND METHODS

Twenty-eight *B. cinerea* isolates were obtained from necrotic tissues on lodgepole pine, Scots pine (*Pinus sylvestris* L.), Engelmann spruce (*Picea engelmannii* Parry), and blue spruce (*Picea pungens* Engelm.) selected from locations throughout greenhouses at three Colorado nurseries (Colorado Hydroponics, Colorado State Forest Service, and Mt. Sopris). Isolations were made on 2 percent water agar and maintained on potato dextrose agar (PDA) slants. Two isolates from

Fig. 1. Sporulation of *Botrytis cinerea* on containerized lodgepole pine.

Fig. 2. Conidiophore of *Botrytis cinerea* isolated from containerized lodgepole pine seedlings. (X450)

California redwood (*Sequoia sempervirens* (D. Don) Endl.) seedlings,[1] one known to be tolerant to benomyl and the other known to be benomyl-sensitive, were used for reference in each of three tests.

Growth tests -- Tolerance to fungicides was determined by assessing radial growth of *B. cinerea* isolates on the surface of PDA amended with fungicides (PDA with no fungicide was used as a check). Benomyl was ground with a mortar and pestle, and all fungicides were suspended in distilled water prior to incorporation into agar. An 8-mm plug of mycelium obtained from the edge of 4-day-old colonies growing on PDA was inoculated onto petri dishes (100 mm diameter) with 25 ml of test media. Each treatment was replicated three (Test 1) or four (Test 2) times, and dishes were incubated in the dark at about 25° C. After 6 days (14 days for isolate 78-19), diameters of the fungal colonies were measured. Data were anlayzed using Tukey's test for multiple comparison of treatment means.

Test 1 - Seven fungicides (Table 1) mixed in PDA at 50 ppm active ingredient (a.i.) were used to test tolerance of *B. cinerea* isolates. Isolates tested included seven from Colorado Hydroponics and benomyl-sensitive (78-18) and tolerant (78-19) isolates from California. Benomyl was added to the media before autoclaving to increase its solubility; the other fungicides were added after media was autoclaved.

Test 2 - This test assessed responses of *B. cinerea* to three fungicides (benomyl, dichloran, chlorothalonil) at different concentrations (5-500 ppm a.i.). Three isolates, chosen for their range of benomyl tolerance, from each of the three nurseries, plus the two California reference isolates were evaluated. All fungicides were added to PDA after autoclaving.

Spore germination tests -- Effects of benomyl on spore germination were determined for *B. cinerea* isolates. Spores were harvested from fungal cultures growing on PDA by flooding petri dishes with 10 ml distilled water and agitating with a sterilized camel's hair paintbrush. Cultures were either 6- or 30-days-old at the time of spore harvest. Spore suspensions were passed through double layers of cheesecloth to remove mycelial fragments, and 1:10

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[1] Provided by Dr. A. H. McCain, Extension Plant Pathologist, University of California, Berkeley.

TABLE 1 Fungicides used to test tolerance of *Botrytis cinerea* isolates (Test 1).

	Chemical Name	Trade Name	Manufacturer
1.	benomyl [Methyl 1 - (butylcarbamoyl) - 2 - benzimidazolecarbamate]	Benlate ® 50 WP	Dupont
2.	benomyl	Benomyl ®	Fertilome
3.	dichloran [2, 6 - Dichloro - 4 - nitroaniline]	Botran ® 75 WP	Tuco (Upjohn)
4.	captan [N - (Trichloromethylthio) - 4 - cyclohexene - 1, 2 - dicarboximide]	Captan ® 50 WP	Stauffer
5.	chlorothalonil [Tetrachloroisophthalo - nitrile]	Daconil 2787 ®	Diamond Shamrock
6.	mancozeb [Manganese and zinc ethylenebisdithiocarbamate]	Dithane ® M-45	Rohm and Haas
7.	zineb [Zinc ethylenebisdithio - carbamate]	Dithane ® Z-78	Rohm and Haas

dilutions were made with distilled water. Approximately 0.5 ml spore suspension was added to each dish and spread uniformly over the agar surface with a sterilized glass rod.

For each isolate tested, one check and two benomyl dishes, each with PDA amended with 50 ppm of the fungicide, were used. After 24 hours incubation at about 24° C, 300 randomly chosen spores on each dish were examined under the compound microscope. Percentage germination on PDA and benomyl-amended media was compared using a one-way analysis of variance.

RESULTS

Isolates of *B. cinerea* obtained from conifer seedlings at three Colorado nurseries were tolerant to benomyl, captan, chlorothalonil, mancozeb, and zineb when grown on PDA-amended media. Only a small percentage of the isolates tested were sensitive to benomyl, which was the most commonly used fungicide by the nurseries. Some *B. cinerea* isolates were tolerant to more than one fungicide.

Growth tests

Test 1 - Benomyl tolerance and sensitivity of the California reference isolates were confirmed in this test (Table 2). Tolerance was evident when linear growth of the fungus was not restricted on fungicide-amended agar. Of the seven Colorado Hydroponics isolates tested, two (78-11, 78-12) showed high levels of tolerance to benomyl, two (78-1, 78-13) were intermediate in response, and three (78-4, 78-6, 78-7) were sensitive as evidenced by little growth on the benomyl-amended agar (Table 2). Response to the two types of benomyl used (Dupont and Fertilome) was significantly different in five of the seven Colorado isolates tested. One isolate (78-19) was more tolerant of Dupont benomyl; whereas, others (78-4, 78-6, 78-7, 78-12) showed greater tolerance to Fertilome benomyl.

Of all the fungicides tested, dichloran was the only one for which no tolerance was shown (Table 2). At least some degree of tolerance to all other fungicides was evident. Tolerance of individual isolates to more than one fungicide was evident. The least effective fungicides were zineb and mancozeb; chlorothalonil was effective against only two isolates (78-4, 78-13). Next to dichloran, captan was most effective in limiting growth of *B. cinerea*.

TABLE 2 Effects of fungicides on radial growth of *Botrytis cinerea* (Test 1). [a]

Fungicide Treatment	*Botrytis cinerea* Isolates								
	California [b]		Colorado Hydroponics						
	78-18	78-19	78-1	78-4	78-6	78-7	78-11	78-12	78-13
Check	100 A	100 A	100 A	100 A	100 A	100 A	100 A	100 C	100 A
Benomyl (Dupont)	1 C	91 A	17 BC	6 C	9 C	8 D	90 AB	98 C	46 BC
Benomyl (Fertilome)	3 C	72 B	31 B	40 B	41 B	40 B	82 AB	122 B	74 AB
Dichloran	0 C	1 D	0 D	0 C	0 C	0 D	0 D	0 F	0 D
Captan	17 B	67 B	8 CD	6 C	12 C	10 CD	9 D	16 E	8 CD
Chlorothalonil	62 B	53 C	23 BC	5 C	19 C	22 C	31 CD	71 D	7 CD
Mancozeb	51 B	99 A	47 A	47 B	49 B	40 B	62 BC	104 C	55 B
Zineb	101 A	101 A	96 A	96 A	99 A	97 A	62 BC	164 A	99 A

a

Fungicide concentration = 50 ppm a.i.; growth expressed as percent of check. Within each isolate, means followed by the same capital letter are not significantly different (P = 0.05) using Tukey's Test for multiple comparison of treatment means.

b

California reference isolates: 78-18 = benomyl sensitive
78-19 = benomyl tolerant

Test 2 - Increasing benomyl concentration resulted in greater inhibition of all isolates (Table 3). Growth patterns displayed by a sensitive (78-1) and tolerant (78-11) isolate are readily apparent in Figures 3 and 4 respectively. Most of the significant differences in growth among treatments occurred at higher benomyl concentrations (158 and 500 ppm a.i.).

All isolates tested were sensitive to 5 ppm of dichloran (Table 3). Increasing fungicide concentrations usually did not significantly alter growth responses.

Inhibition by chlorothalonil generally increased with greater fungicide concentration. Ten of the eleven isolates tested were tolerant to the fungicide at 5 ppm a.i. Significant decreases in growth usually occurred between fungicide treatments of 5 ppm and the higher concentrations (50 and 500 ppm a.i.). Most isolates displayed tolerance to both benomyl and chlorothalonil. Some isolates (78-18, 78-29) were sensitive to benomyl and tolerant to chlorothalonil, whereas, one isolate (78-27) was tolerant to benomyl and sensitive to chlorothalonil.

Spore germination tests -- Benomyl did not restrict initial spore germination of the *B. cinerea* isolates (Table 4). In fact, six of the isolates showed significantly greater germination in the benomyl-amended agar.

Response of germ tubes provided additional evidence of fungicide tolerance. Germ tubes from spores of benomyl-sensitive isolates (Table 4) often lysed or wrapped around the spore (Fig. 5), whereas germ tubes from benomyl-tolerant isolates grew over the agar surface with no apparent inhibition (Fig. 6).

One-way analysis of variance indicated that a significantly greater percentage of spores from 6-day-old cultures germinated than spores from 30-day-old cultures. Isolates varied in germinability.

DISCUSSION

The development of tolerance to fungicides is mainly due to the intense selection pressure exerted on resident populations of the pathogen by heavy applications of a single fungicide (18). When fungal pathogen populations are subjected to fungicides, sensitive individuals are selectively killed. Those rare individuals, which

TABLE 3 Effects of benomyl, dichloran and chlorothalonil on radial growth of *Botrytis cinerea* (Test 2). [a]

Fungicide Treatment	Conc. (ppm)	*Botrytis cinerea* Isolates: California [b] 78-18	California [b] 78-19	Colorado Hydroponics 78-1	Colorado Hydroponics 78-4	Colorado Hydroponics 78-11	Colo. St. For. Serv. 78-20	Colo. St. For. Serv. 78-27	Colo. St. For. Serv. 78-29	Mt. Sopris 78-36	Mt. Sopris 78-38	Mt. Sopris 78-39
Check	-	100 A	100 A	100 A	100 A	100 A	100 A8	100 A	100 A	100 AB	100 A	100 AB
Benomyl	5.0	3 EF	63 BC	98 A	76 8	96 A	107 A	104 A	2 E	102 A	101 A	103 A
	15.8	2 EF	69 AB	48 C	13 D	110 A	97 A8	102 A	1 EF	82 C	100 A	99 AB
	50.0	3 EF	83 AB	9 F	4 D	92 A	98 AB	96 A	2 EF	86 BC	93 B	96 B
	158.0	1 EF	59 BCD	3 F	4 D	65 B	77 BC	81 B	1 EF	64 D	63 C	62 C
	500.0	0 F	26 DE	3 F	1 D	33 CD	61 C	48 C	0 F	47 EF	43 E	45 E
Dichloran	5.0	7 E	6 E	11 EF	10 D	7 E	11 E	7 DE	6 E	10 G	2 F	7 G
	15.8	1 EF	2 E	0 F	0 D	0 E	0 E	0 E	1 EF	0 G	0 F	1 H
	50.0	2 EF	2 E	3 F	3 D	1 E	1 E	13 D	2 EF	1 G	6 F	1 H
Chlorothalonil	5.0	54 8	46 CD	73 8	77 B	43 C	44 D	11 DE	50 B	60 DE	58 C	61 C
	50.0	30 C	53 BCD	42 CD	44 C	16 DE	9 E	9 DE	30 C	41 F	57 D	52 D
	500.0	18 D	31 DE	28 DE	35 C	12 E	11 E	6 DE	22 D	38 F	41 E	39 F

[a] Growth expressed as percent of check. Within each isolate for all fungicide treatments, means followed by the same capital letter are not significantly different (P = 0.05) using Tukey's Test for multiple comparisons of treatment means.

[b] California reference isolates: 78-18 = benomyl sensitive
78-19 = benomyl tolerant

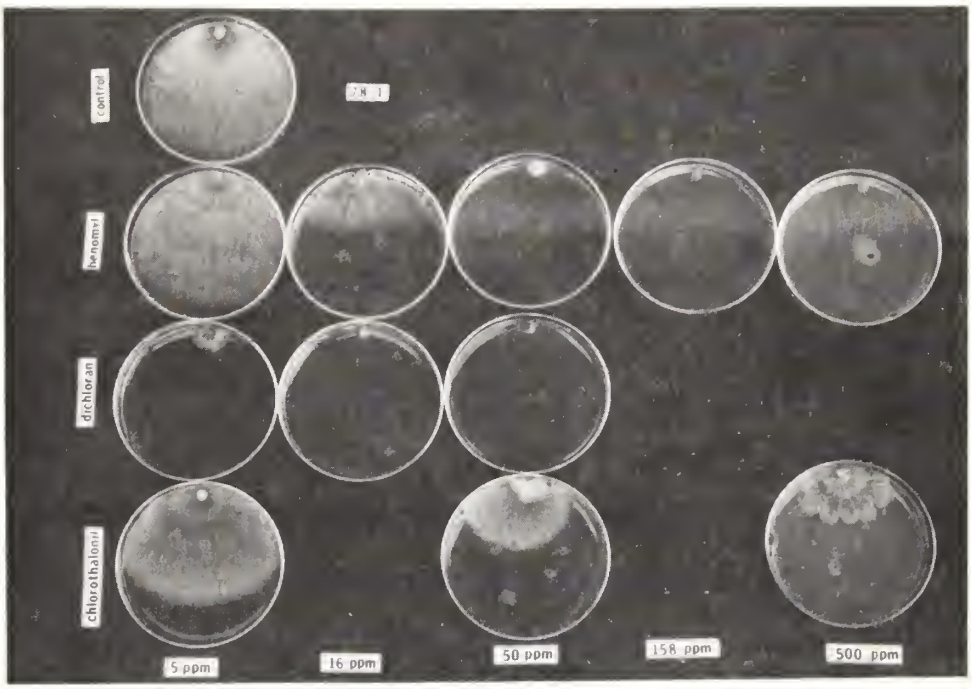

Fig. 3. Growth habit of *Botrytis cinerea* isolate 78-1 on fungicide-amended agar.

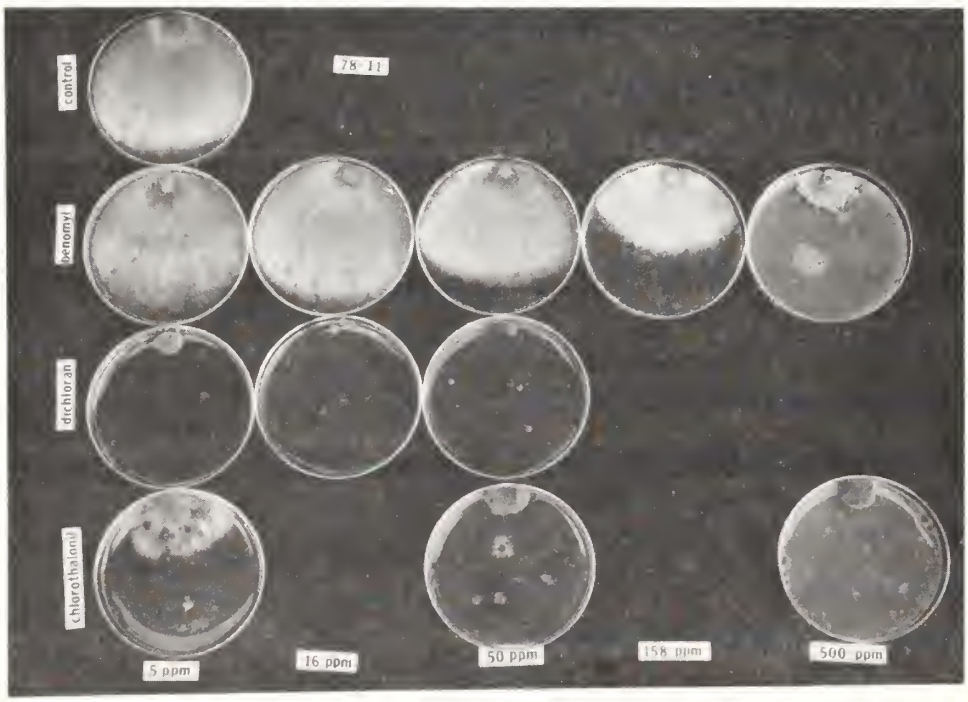

Fig. 4. Growth habit of *Botrytis cinerea* isolate 78-11 on fungicide-amended agar.

TABLE 4 Effects of benomyl on germination of *Botrytis cinerea* spores.

Isolate Group [a]	Isolate	Percentage Germination				Benomyl Tolerance Class [e]
		Check [b]	Benomyl (500 ppm) [c]	F values [d]		
I	78-1	87.7	87.0	0.12	NS	S
	78-11	95.2	95.2	0.00	NS	T
	78-18	91.6	96.8	58.43	**	S
	78-20	93.6	95.6	5.18	NS	T
	78-27	95.8	96.9	1.77	NS	T
	78-29	93.5	93.7	0.30	NS	S
	78-36	93.3	94.8	0.56	NS	T
	78-38	93.2	95.6	9.83	*	T
	78-39	79.5	80.4	0.12	NS	T
Average (Isolate Group I)		91.5	92.9			
II	78-2	58.4	57.5	0.08	NS	T
	78-3	84.2	92.3	10.46	*	S
	78-4	85.1	94.0	43.04	**	S
	78-7	77.3	96.3	107.89	**	S
	78-16	54.1	54.9	0.48	NS	T
Average (Isolate Group II)		71.8	79.0			

a
Isolate Groups: I = from 6-day-old cultures
II = from 30-day-old cultures

b
Average values from 300 examined spores

c
Average values from 600 examined spores

d
Based on one-way analysis of variance comparing check and benomyl treatments
NS = Not statistically significant
* = Statistically significant (P = 0.05)
** = Statistically significant (P = 0.01)

e
T = benomyl tolerant based on growth of germ tubes over agar surface
S = benomyl sensitive based on germ tube lysis and restricted growth

Fig. 5. Germ tube habit of benomyl-sensitive isolate (78-7) upon conidial germination on benomyl-amended agar. (X450)

Fig. 6. Germ tube habit of benomyl-tolerant isolate (78-11) upon conidial germination on benomyl-amended agar. (X450)

by chance harbor a mutated gene for tolerance, may multiply without competition from the normal wild population (6). This new, tolerant population then becomes dominant, and crop losses can occur despite continued fungicide application.

In two Colorado nurseries sampled (Colorado Hydroponics and Mt. Sopris), tolerance to benomyl existed even though benomyl had not been previously used. Tolerance either developed rapidly (within one month at Colorado Hydroponics and one year at Mt. Sopris) or resident *B. cinerea* populations (sources of initial infection) were benomyl tolerant.

All isolates tested were sensitive to dichloran, even at low concentrations. No field resistance to dichloran has been reported for *Botrytis* spp. (18); although there is one report (11) of resistance developed by *Sclerotium cepivorum* on onions. This fungicide provides a possible alternative for *B. cinerea* control in Colorado nurseries. However, use of a single fungicide is discouraged because the pathogen rapidly develops tolerance (10, 17).

The performance of chlorothalonil was disappointing compared to its reported effectiveness on *Botrytis cinerea* (4), *B. squamosa* (12) and *Cercospora arachidicola* (10) strains tolerant to benomyl. Although this fungicide has been recommended as an alternative to benomyl for *B. cinerea* control on containerized conifers (16), our results indicate that tolerance in fungus populations tested may preclude its usefulness in certain Colorado nurseries.

Captan was generally effective against the isolates tested. This non-systemic organic compound has a history of good performance (18) and may be considered an alternative to benomyl and other systemic fungicides for which tolerance may develop (18, 20).

Fungicide-tolerant strains displayed decreasing tolerance with increasing fungicide concentration. This agrees with results found by other investigators (13, 17). However, growth of three sensitive isolates was restricted throughout the fungicide concentration spectrum. From a practical standpoint, heavy fungicide applications will probably have little effect in overcoming tolerance. Such applications will most likely increase selection pressure and result in the development of more tolerant strains of the fungus.

Benzimidozole fungicides are believed to inhibit mycelial growth rather than spore germination (25). Benomyl did not reduce spore germination of *B. cinerea* isolates tested. Rather, the chemical affected germ tubes by causing lysis and abnormal growth in benomyl-sensitive isolates. Germ tube growth and development

were not affected by benomyl in *B. cinerea* strains tolerant to the chemical. Similar effects of benomyl on conidial germ tubes was reported by Richmond and Phillips (19) for *B. cinerea* and Davidse (5) for *Aspergillus nidulans*.

CONCLUSIONS

Based on the results of this investigation, the following recommendations are proposed to reduce losses from *B. cinerea* on containerized conifer seedlings:

1. Promote cultural methods that are unfavorable to fungus buildup including greater aeration and spacing among seedlings, modification of watering schedules to reduce moisture retention on foliage, and sanitation practices to reduce potential inoculum.

2. Avoid the exclusive use of a single fungicide for extended periods and apply fungicides at the lowest possible rates to achieve satisfactory control.

3. Integrate fungicides with different modes of action into schedules to avoid the possibility of development of tolerance to several fungicides.

4. Establish and maintain a program to detect chemical tolerance by screening isolates on fungicide-amended agar. Such a program will provide necessary information for adjusting fungicide schedules.

This publication reports research involving pesticides. It does not contain recommendations for their use, nor does it imply that the uses discussed have been registered. All uses of pesticides must be registered by appropriate State and/or Federal agencies before they can be recommended.

CAUTION: Pesticides can be injurious to humans, domestic animals, desirable plants, and fish or other wildlife -- if they are not handled or applied properly. Use all pesticides selectively and carefully. Follow recommended practices for the disposal of surplus pesticides and pesticide containers.

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